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Plenary Article

Possible involvement of activated locus coeruleus–noradrenergic neurons in pain-related sleep disorders

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HIGHLIGHTS

- Activation of LC noradrenergic neurons increases in wakefulness with decreased NREM.
- Neuropathic pain increases the activity of LC–PFC noradrenergic neurons.
- Activation of LC–PFC noradrenergic neurons facilitates sleep-to-wake transitions.

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ABSTRACT

The locus coeruleus (LC) is a noradrenergic brainstem structure that is considered to play a role in promoting arousal. To further clarify the role of LC noradrenergic neurons, we performed an optogenetic assay by injecting AAV-channelrhodopsin-2 (ChR2) into the LC of cre-tyrosine hydrolase (TH) mice. We found here that the specific activation of LC noradrenergic neurons produced a significant increase in wakefulness and a significant decrease in non-rapid eye movement (NREM) sleep during photostimulation. On the other hand, neuropathic pain is believed to significantly interfere with sleep, and inadequate sleep may contribute to the stressful negative consequences of living with pain. In the present study, sciatic nerve ligation, which produced significant thermal hyperalgesia, significantly increased the levels of noradrenaline released in the prefrontal cortex (PFC) by the weak electrical stimulation of neurons in the LC. Under these conditions, the systemic administration of adrenaline α and β inhibitor cocktail at 7 days after sciatic nerve ligation restored the increased wakefulness and decreased NREM sleep to normal levels. These results suggest that neuropathic pain may accelerate neurons in the LC, and its overactivation may be, at least in part, associated with sleep disturbance under neuropathic pain.

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1. Introduction

The locus coeruleus (LC) is a noradrenergic brainstem structure that is thought to play a major role in promoting arousal [1]. LC neurons fire during wakefulness, decrease firing during non-rapid eye movement (NREM) sleep, and are virtually silent during rapid eye movement (REM) sleep [2]. It has been reported that the level of mRNA for norepinephrine transporter (NET) was increased in rats that were deprived of REM sleep for 3 days or longer [3].

Neuropathic pain can be very difficult to treat, and the presence of comorbidities, such as poor sleep and mood disorders, can greatly affect the outcome. Clinical studies have shown that most patients experience problems with sleep after they develop chronic pain [4]. In our previous study, we demonstrated that neuropathic pain accelerated the activity of dorsal raphe nucleus (DRN)-serotonergic neurons, implying that the activation of DRN neurons may play a role in sleep dysregulation under a neuropathic pain-like state [5]. However, the neurophysiological mechanism by which neuropathic pain affects sleep-arousal patterns remains unclear. Therefore, in this study, we investigated the role of the LC-prefrontal cortex (PFC) noradrenergic system in sleep dysregulation in mice with neuropathic pain.

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2. Methods

2.1. Ethics statement

All experiments were conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals at Hoshi University.

2.2. Animals

Male C57BL/6J mice (8 weeks old; Tokyo Laboratory Animals Science, Tokyo, Japan) and tyrosine hydroxylase (TH)-cre transgenic mice (B6. Cg-Tg(Th-cre) 1Tmd/J) (Jackson Laboratory) were used for this study. TH is the rate-limiting enzyme in the synthesis of noradrenaline and dopamine. Animals were housed in a room maintained at $23 \pm 1^\circ\text{C}$ with a 12 h light–dark cycle. Food and water were available *ad libitum*.

2.3. Virus preparation

We purchased AAV-FLEX-rev-ChR2 (H134R)-mCherry from Addgene (Plasmid 18916). The recombinant AAV vectors were serotyped with AAV5 coat proteins and packaged by a viral vector core, which was provided by Drs. Ryosuke Matsui and Dai Watanabe (University of Kyoto). The final viral concentration was 2×10^{13} particles/mL. Aliquots of virus were stored at -80°C before stereotaxic injection. AAV viruses were injected in the bilateral LC (from the bregma: antero-posterior, -5.4 mm ; mediolateral, $\pm 1.28\text{ mm}$; and dorsal, -3.6 mm) using a microsyringe pump ($0.25\text{ }\mu\text{L/min}$, 4 min).

2.4. Immunohistochemistry

TH-Cre transgenic mice on day 14 after the injection of AAV were deeply anesthetized and perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate-buffer (PB, pH 7.4). After perfusion, the brain was removed and thick coronal blocks of the brainstem including the LC were rapidly dissected and fixed in 4% paraformaldehyde for 2 h. They were then permeated with 20% sucrose in 0.1 M PBS for 1 day and 30% sucrose in 0.1 M PBS for 2 days with agitation. The brain sections were stored at -30°C . Transverse sections were cut with a cryostat (Leica CM1510; Leica Microsystems, Heidelberg, Germany) at a thickness of $8\text{ }\mu\text{m}$ and thaw-mounted on poly-L-lysine-coated glass slides. Brain sections that included the LC were incubated in blocking solution, 3% normal goat serum (NGS; Vector Laboratories, Inc., USA) in 0.01 M PBS, for 1 h at room temperature, and then incubated for 48 h at 4°C with primary antibodies diluted in 3% NGS: anti-TH (mouse monoclonal, ImmunoStar, Inc., Hudson, WI, USA). The antibody was then rinsed with PBS and incubated with an appropriate secondary antibody conjugated with AlexaTM 488 (Molecular Probes, Inc.) for 2 h at room temperature. The fluorescence of immunolabeling was detected using a light microscope (BX-61, Olympus, Inc., Tokyo, Japan) and digitized images.

2.5. Photostimulation

An 8 mm unilateral cannula (EIM-330; Eicom) was surgically implanted at least 1 day before the experiment. The cannula was placed above the LC and fixed to the skull with quick self-curing acrylic resin. An optical fiber ($50\text{ }\mu\text{m}$ diameter; Lucir) was placed inside the cannula at least 2 h before the stimulation experiments. The light source was a 473 nm blue laser (Lucir; COME2-LB473 model, Japan) that was controlled by an electronic stimulator (Nihon Kohden, Japan) to generate tonic light pulses: interval (323 ms), duration (10 ms), main interval (1 s), train (3). The power

output was measured at the tip of the fiber with a light meter (NOVA, Ophir, Japan) when the laser was activated in continuous mode.

2.6. In vivo microdialysis and high-performance liquid chromatography

A microdialysis probe (D-1-6-01; Eicom, Japan) was inserted directly into the PFC (from the bregma: anteroposterior, $+1.5\text{ mm}$; mediolateral, $+0.5\text{ mm}$; dorsoventral, -3.7 mm) according to an atlas of the mouse brain. The probe was fixed to the skull with quick self-curing acrylic resin. The probes were perfused continuously ($1\text{ }\mu\text{L/min}$) with artificial cerebrospinal fluid: 0.9 mM MgCl_2 , 147.0 mM NaCl , 4.0 mM KCl , and 1.2 nM CaCl_2 . Outflow fractions were collected, and mice were subjected to photostimulation or electrostimulation for 15 min. Dialysis samples were collected for 1 h after stimulation and analyzed by high-performance liquid chromatography with electrochemical detection (HTEC-500; Eicom). Noradrenaline was separated by column chromatography and identified and quantified by the use of a standard (Sigma–Aldrich, Inc., USA).

2.7. Electroencephalogram and electromyogram recordings

Electroencephalogram (EEG) and electromyogram (EMG) electrodes were implanted for polysomnographic recordings (Pinnacle Technology, Inc., USA). For monitoring EEG signals, two stainless-steel EEG recording screws were positioned 1 mm anterior to the bregma or lambda, both 1.5 mm lateral to the midline. Stainless-steel, Teflon-coated wires were placed bilaterally into both trapezius muscles to monitor EMG activity. We began to monitor the sleep/wake state 2 days after placement of the EEG recording screws. EEG/EMG signals were amplified, filtered (EEG, $0.5\text{--}30\text{ Hz}$; EMG, $20\text{--}200\text{ Hz}$), and digitized at a sampling rate of 128 Hz . The data were subjected to an analysis by SLEEPSIGN software (Kissei Comtec, Japan). Vigilance was automatically classified off-line into three stages using 5-s epochs: wakefulness, rapid eye movement (REM) and non-REM (NREM) sleep. For each epoch, the EEG power density in the δ ($0.75\text{--}4.0\text{ Hz}$) and θ waves ($6.25\text{--}9.0\text{ Hz}$) and the integrated EMG value were displayed on a PC monitor. For each 5-s epoch, the vigilance state was determined to be either wakefulness (high EMG and low EEG amplitude and high theta activity concomitant with highest EMG values), NREM sleep (low EMG and high EEG amplitude, high delta activity) or REM sleep (low EMG and low EEG amplitude, high theta activity), and the scores were manually entered into a PC using a keyboard. EEG and EMG activities were measured 7 days after sciatic nerve ligation.

2.8. Neuropathic pain model

Mice were anesthetized with 3% isoflurane. A partial sciatic nerve ligation model was produced as described previously [6]. Briefly, the sciatic nerve on the right side (ipsilateral side) was ligated by tying a 8–0 silk suture around approximately one-half its diameter under a light microscope (SD30, Olympus, Tokyo, Japan). In sham-operated mice, the nerve was only exposed without ligation.

2.9. Measurement of thermal thresholds

The right plantar surface of mice was subjected to a well-focused, radiant heat light source (model 33 Analgesia Meter; IITC/Life Science Instruments, Woodland Hills, CA) to assess its sensitivity to thermal stimulation. Quick movements of the hind paw away from the stimulus, regardless of licking of the hind paw, were considered to be a withdrawal response.

2.11. Administration of adrenaline α and β inhibitors

Five days after sciatic nerve ligation or sham operation, mice were equipped with a head-mount for the measurement of EEG and EMG to assess the sleep cycle, and then connected to a head cable and recording device one day before the EEG and EMG recording. Seven days after sciatic nerve ligation, mice were systemically co-administered 10 mg/kg of phentolamine and 10 mg/kg of propranolol, and EEG and EMG were measured for 3 h after the injection.

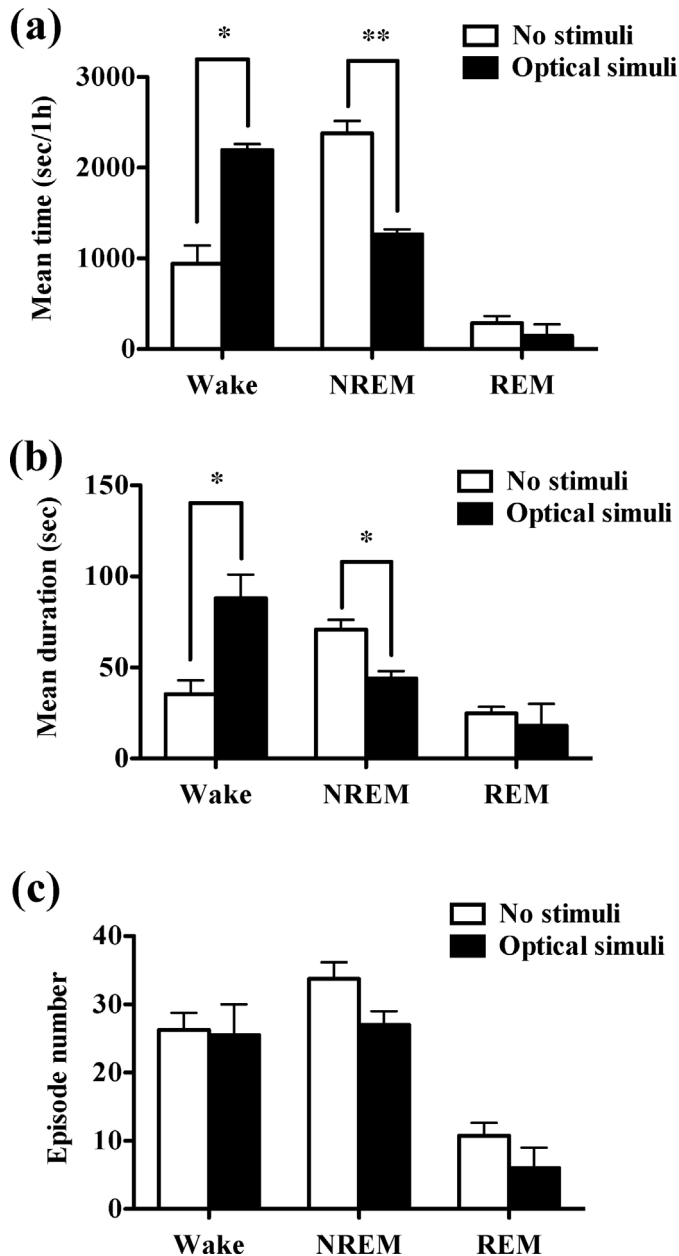


Fig. 2. Changes in the sleep/wake pattern after optical stimulation in the LC. Using an optogenetic tool, we examined the effect of prolonged photostimulation of the PFC (8 mW, 3 Hz, 473 nm, blue light for 1 h) on sleep-wake patterns during the inactive period in TH-Cre transgenic mice in which AAV-ChR2 had been injected into the LC. ((a)–(c)) Mean time (a), mean duration (b), and number of episodes (c) were determined from EEG/EMG recordings for 3-h periods. Values are shown as the means with SEM of 3 mice. * $p < 0.05$ and ** $p < 0.01$ vs. No stimuli group (Student's t -test).

2.12. Statistical analysis

Data are expressed as the mean with S.E.M. The statistical significance of differences between groups was assessed by Student's t -test.

3. Results

3.1. Specific expression of optogenetic transgenes in LC neurons of TH-cre transgenic mice

Cre-inducible recombinant AAV was used to genetically target ChR2 expression to noradrenergic neurons in the LC. Double-floxed reverse ChR2-mCherry cassette was packaged in AAV vectors and serotyped with AAV5 coat proteins to produce high-titer virus preparations (Fig. 1a). We observed a high density of mCherry expression in the LC area (Fig. 1b). To confirm the co-localization of ChR2-mCherry and TH in the LC, double-labeling experiments were performed. As a result, TH-IR was mostly observed in cells that reacted with ChR2-mCherry (Fig. 1c and d).

3.2. Changes in the increased levels of noradrenaline in dialysate of the PFC induced by photostimulation of the LC

In an *in vivo* microdialysis study, the levels of noradrenaline in dialysate of the PFC of TH-cre mice in which AAV-ChR2 had been injected into the LC were significantly increased by photostimulation of the LC (Fig. 1e, ** $p < 0.01$ vs. No stimuli).

3.3. Photostimulation of the LC of TH-Cre transgenic mice that had been injected with AAV-ChR2 caused sleep–arousal transitions

Using an optogenetic tool, we examined the effect of photostimulation of the LC (8 mW, 3 Hz, 473 nm, blue light for 1 h) on sleep-wake patterns during the inactive period. TH-Cre transgenic mice in which AAV-ChR2 had been injected into the LC showed a significant increase in wakefulness and a significant decrease in NREM sleep (Fig. 2a, * $p < 0.05$, ** $p < 0.01$ vs. No stimuli) during photostimulation, compared to mice without stimulation (Fig. 2a). The mean duration of wakefulness was significantly increased, and the mean duration of NREM sleep was significantly decreased during photostimulation in these mice (Fig. 2b, * $p < 0.05$). There were no differences in the number of episodes of any of the stages (Fig. 2c).

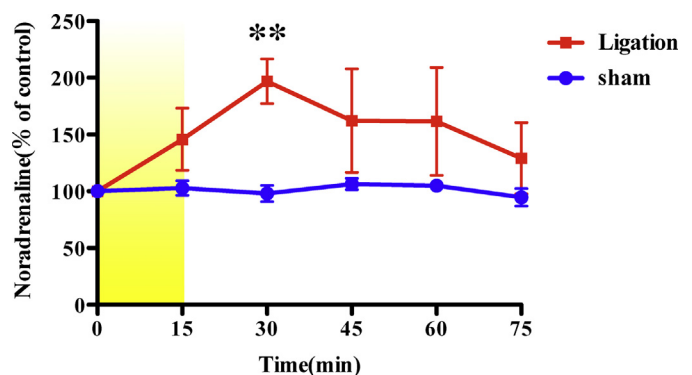


Fig. 3. Changes in the levels of noradrenaline in dialysate induced by electrical stimulation of the LC at 7 days after sciatic nerve ligation. To investigate the effect of weak electrical stimulation of the LC, we evaluated the levels of noradrenaline in dialysate of the PFC in mice by *in vivo* microdialysis. The mice were subjected to weak electrical stimulation (sine-wave pulses, 100 Hz, 150 μ A) for 15 min. The data are expressed as a percentage of the corresponding baseline levels \pm SEM of 3 mice. ** $p < 0.01$ vs. sham group (Student's t -test).

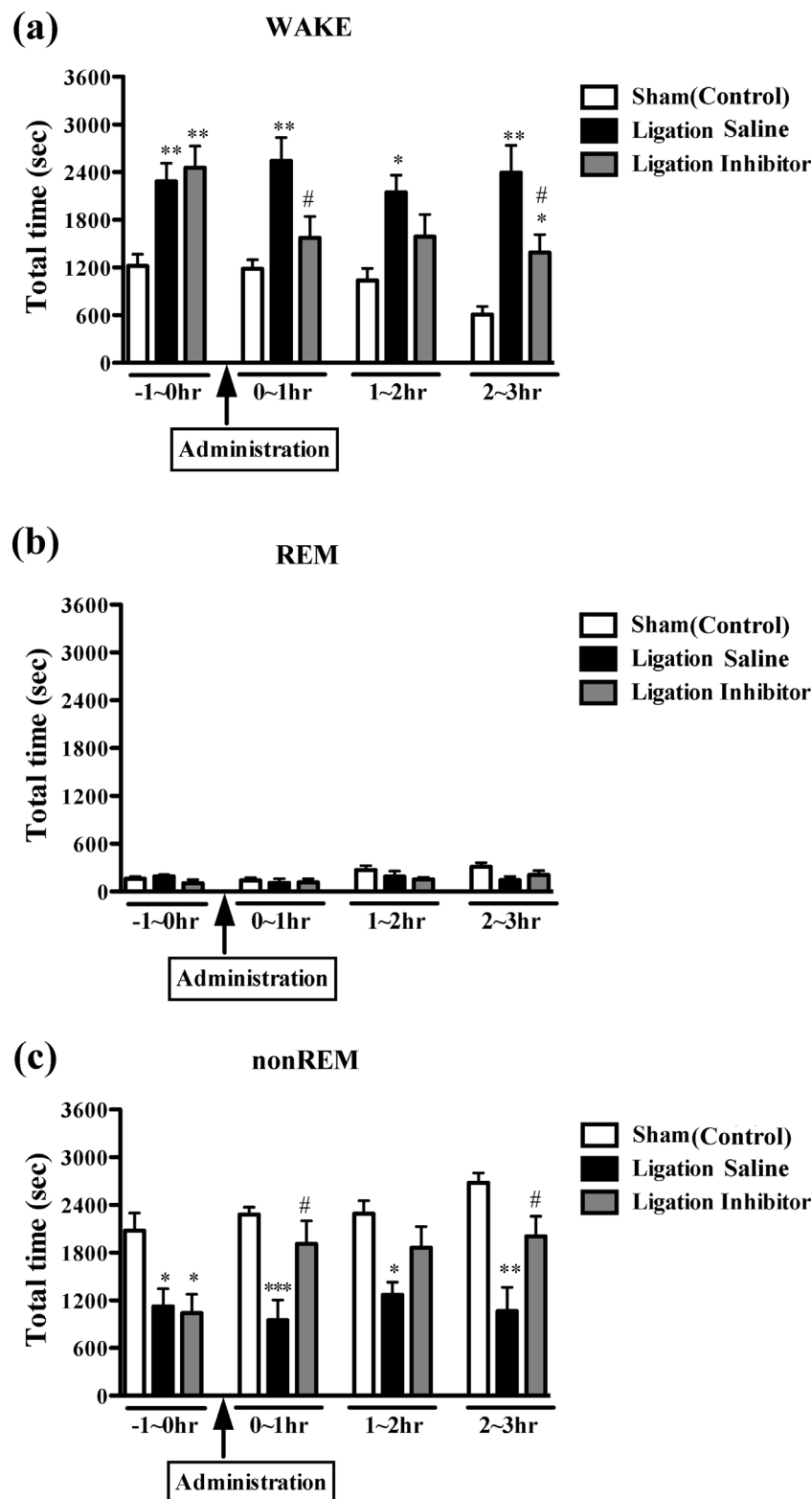


Fig. 4. Effects of administration of adrenaline α and β inhibitors on sleep disorders at 7 days after sciatic nerve ligation.

Mice were administered adrenaline α and β inhibitor cocktail (10 mg/kg phentolamine and 10 mg/kg propranolol) or saline at 7 days after sciatic nerve ligation or sham operation (control). The total mean times (s/h) spent in wakefulness (a), REM sleep (b), and NREM sleep (c) stage were measured by EEG/EMG recordings for 3 h after administration and 1 h before administration. Each point represents the mean \pm SEM of 5 mice. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. sham group, # p < 0.05 vs. ligation-saline group (Student's t -test). Sham (control): Sham operation + α/β inhibitor treatment.

3.4. Increased level of noradrenaline in the PFC of mice with sciatic nerve ligation by weak electrical stimulation of the LC

We confirmed that ligation of the sciatic nerve markedly decreased the latency of paw withdrawal in response to thermal stimuli on the ipsilateral side (from 7.9 ± 1.1 s for the sham-operated group to 5.1 ± 0.4 sec for the sciatic nerve-ligated group; figure not shown). Under these conditions, the level of noradrenaline in dialysate of the PFC of sciatic nerve-ligated mice was significantly increased at 30 min after the weak electrical stimulation of LC cells for 15 min with no increase in the level of noradrenaline in dialysate of the PFC of sham-operated mice (Fig. 3, $**p < 0.01$ vs. sham group), indicating that LC–PFC noradrenergic neurons can be sensitized by sciatic nerve ligation.

3.5. Effects of systemic administration of adrenaline α and β inhibitors on the sleep disorder in mice with sciatic nerve ligation

We finally demonstrated the changes in the sleep/wake pattern after sciatic nerve ligation in the presence or absence of adrenaline α and β inhibitors. Seven days after sciatic nerve ligation or sham operation, mice were systemically co-administered 10 mg/kg of phentolamine and 10 mg/kg of propranolol or saline, and EEG and EMG were measured for 3 h after the injection during inactive “light” periods. Mice with sciatic nerve ligation and saline treatment showed a significant increase in wakefulness (Fig. 4a, $**p < 0.01$ vs. sham group) and a significant decrease in NREM sleep (Fig. 4c, $*p < 0.05$ vs. sham group) during this period. In contrast, REM sleep was not affected by sciatic nerve ligation (Fig. 4b). Under these conditions, systemic administration of the adrenaline α and β inhibitor cocktail (10 mg/kg of phentolamine plus 10 mg/kg of propranolol; the doses of both drugs can block central-mediated behavioral responses, respectively, [7]) at 7 days after sciatic nerve ligation significantly inhibited the increased wakefulness and decreased NREM sleep to the levels in the sham control (Fig. 4, $\#p < 0.05$ vs. ligation-saline group).

4. Discussion

In a previous study, we found that sciatic nerve-ligated mice exhibited sleep disturbance along with thermal hyperalgesia [5]. Although it has been considered that several areas in the brainstem and forebrain are important for modulation and expression of the sleep/wake cycle [8], and several neurotransmitters and neuropeptides are involved in modulation of this cycle [9–11], the neurophysiological and molecular mechanisms by which neuropathic pain affects sleep/arousal patterns are not fully understood. The LC is a dense collection of noradrenaline-containing neurons in the dorsolateral pontine tegmentum, and is thought to play a major role in promoting both tonic and phasic processes of arousal [1,12]. It has also been recognized that LC–noradrenergic neurons are crucial for switching between sleep and wakefulness [12]. LC neurons have been shown to fire during wakefulness, decrease firing during NREM sleep, and to be silent during REM sleep [2]. However, few studies have examined whether the activity of noradrenergic neurons in the LC is affected by neuropathic pain. In our preliminary study, we found that ligation of the sciatic nerve significantly increased the expression of noradrenaline transporter in the LC of mice (data not shown), suggesting that the activity of LC–noradrenergic neurons is likely to be affected by neuropathic pain. To clarify the possible changes in the activity of noradrenergic neurons in the LC under a neuropathic pain-like state, we first performed an *in vivo* microdialysis study. The level of noradrenaline in dialysate of the PFC in nerve-ligated mice was significantly greater than that in sham-operated mice after weak electrical stimula-

tion of the LC. Although the causal mechanism of this event is still unclear, these findings suggest that neuropathic nociception may result in the increased activity of noradrenergic neurons that project from the LC to the PFC, eventually leading to a change in brain dynamics.

A major advantage of the optogenetic approach is its ability to elicit neuronal activity within specific temporal windows with minimal disturbance to the animal [13]. In contrast, electrical stimulation is likely to affect other neurons and glial cells around the target, since mice have a very small LC. The optogenetic gain-of-function approach can be used to achieve not only region-specific effects but also the efficient genetic targeting of noradrenergic neurons, which may explain the difference between the results with the optogenetic approach and those in other studies [14]. In the present study, we performed a system function analysis of LC–PFC noradrenergic neurons by injecting AAV–ChR2 into the LC of TH–cre mice to determine whether the activation of LC–PFC noradrenergic neurons is necessary for interference with sleep. First, we performed an immunohistochemical analysis to evaluate the expression of ChR2 in noradrenergic neurons in the LC. Although our experiment on the immunostaining of LC neurons in acute brainstem slices showed ChR2 expression in LC–noradrenergic neurons, we cannot conclude whether these tools are as precise and as effective at modulating neuronal activity *in vivo*. Therefore, we next performed an *in vivo* microdialysis study to evaluate the levels of noradrenaline released in the PFC during photostimulation of the LC. The levels of noradrenaline in dialysate of the PFC in TH–cre mice in which AAV–ChR2 had been injected into the LC were significantly increased by photostimulation of the LC. Using this strategy, we assessed changes in the sleep and arousal pattern using EEG and EMG during prolonged photostimulation of the LC expressing ChR2. TH–cre transgenic mice in which AAV–ChR2 was injected into the LC showed a significant increase in wakefulness and a significant decrease in NREM sleep. The mean duration of wakefulness was significantly increased, while the mean duration of NREM sleep was significantly decreased during photostimulation in these mice. Under the present conditions, there were no differences in the number of episodes of any of the stages. These results suggest that the activation of LC–PFC noradrenergic neurons, which could be induced under a neuropathic pain-like state, may be sufficient for promoting sleep/wake transitions.

We have demonstrated that neuropathic pain can increase the activity of LC–PFC noradrenergic neurons. Using an optogenetic approach, we confirmed that the activation of LC–PFC noradrenergic neurons facilitates sleep-to-wake transitions. This phenomenon may, at least partly, explain the sleep disturbance that is observed under neuropathic pain. This contention can be strongly supported by the present finding that a neuropathic pain-like state produced by sciatic nerve ligation was associated with a significant increase in wakefulness and a significant decrease in NREM sleep, and these effects were inhibited by the combined systemic injection of α - and β -adrenergic blockers. It has been reported that both α and β receptors are distributed in the PFC [15,16]. Although further studies are needed using an optogenetic tool with TH–cre transgenic mice in which AAV–HAL (a light-driven chloride pump) is injected into the LC, we propose that the acceleration of LC–PFC noradrenergic neurons, as well as DRN–5-HTergic neurons [5] may, at least in part, explain the sleep disturbance under a state of neuropathic pain.

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